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Fluorescent In Situ Hybridization and Microautoradiography Applied to Ecophysiology in Soil

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Soil microbial communities perform many important processes, including nutrient cycling, plant–microorganism interactions, and degradation of xenobiotics. The study of microbial communities, however, has been limited by cultural methods, which may greatly underestimate diversity. The advent of nucleic acids technologies allows microbial communities to be quantified and classified without the limitations of cultivation. Fluorescent in situ hybridization (FISH) and other tools of molecular ecology are now being used to investigate community structure and diversity of soils, aquifers, and other natural habitats. Based on these studies, soil microbial communities are diverse and appear to respond to anthropogenic inputs, such as fertilizer, manure, and pollutants, as well as the more well-known constraints imposed by temperature and moisture. Yet most nucleic-acids-based technologies are unable to directly link phylogeny with processes in a manner similar to cultivation-based approaches, restricting the conclusions that can be drawn from the large data sets they generate. Recently, the combination of FISH with microautoradiography (FISH-MAR) allows cells active in processes to be quantified and simultaneously classified phylogenetically. In this review, we discuss how FISH-MAR can be used to quantify the specific microbial phylotype(s) responsible for a microbially catalyzed process. Examples of the use of FISH and FISH-MAR in soils and sediments are described. The capabilities and limitations of these techniques for linking microbial community structure and function are discussed.

Abbreviations: CLSM, confocal laser scanning microscopy; FISH, fluorescent in situ hybridization; MAR, microautoradiography; PCR, polymerase chain reaction.

Much of our understanding of microbial ecology has stemmed from the study of microorganisms cultivated from diverse natural environments. Accurate descriptions of the complex metabolic interactions within these diverse systems based on data obtained using only cultivated microorganisms has proven problematic, however. This may be rooted in the fact that only a small fraction of natural microbial communities (0.1–10%) have been successfully cultivated (Winogradsky, 1949; Ward et al., 1992; Amann et al., 1995; Head et al., 1998; Janssen, 2006). Advances in molecular microbial ecology within the last 20 yr have provided means by which the uncultivated fraction of natural microbial communities can be identified. Whole groups of uncultivated and uncharacterized microorganisms, known only from their nucleic acid sequences, have emerged as significant members in many environments. The relationship between in situ physiology and microbial community remains ambiguous in terrestrial ecosystems.

Fluorescent in situ hybridization (FISH) is one of many nucleic acids techniques useful for studying microorganisms in their natural environments. The technique, first applied by DeLong et al. (1989), uses the ability of dye-labeled oligonucleotide probes to selectively bind to the bacterial ribosome. The dyed cells can then be counted with epifluorescence microscopy, confocal laser scanning microscopy (CLSM), or flow cytometry. Application of these procedures with multiple oligonucleotide probes can provide a general picture of microbial diversity. Alternatively, the use of highly specific probes can provide information on one or several species. The use of 16S-rRNA FISH and related approaches have been reviewed previously (Amann et al., 1995; Moter and Göbel, 2000). Probes are available in hierarchical sets from the domain level (Bacteria and Archaea) to the species level based on the 16S rDNA database.

Microautoradiography (MAR) is a process by which radioactive materials taken up by cells are visualized by exposure of a thin film of photographic emulsion on a microscope slide. The exposed emulsion creates a pattern of silver grains surrounding the cells that can be seen clearly by transmission light microscopy. Recently, two independent research groups introduced the coupling of FISH with microautoradiography (FISH-MAR) facilitating the phylogenetic identification of substrate-active cells within complex microbial communities (Lee et al., 1999; Ouverney and Fuhrman, 1999). This is a significant advancement over earlier MAR techniques based on morphology, general staining, or antibody staining because it allows investigators to answer questions regarding the metabolic state of microbial communities without a priori knowledge of the microbial community of interest, or when morphology and phylogeny may not reflect well cellular physiology (Fliermans and Schmidt, 1975; Meyer-Reil, 1978; Fuhrman and Azam, 1982; Tabor and Neihof, 1982, 1984; Andreasen and Nielsen, 1997). The FISH-MAR technique has proven useful for the study of diverse environments including marine and fresh waters and sediments, hot-spring microbial mats, and contaminated aquifer sediments (Lee et al., 1999; Ouverney and Fuhrman, 1999; Otte et al., 1999; Gray et al., 2000; Nübel et al., 2002; Ito et al., 2002; Glaeser and Overmann, 2003;

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Malmstrom et al., 2004; Villa et al., 2004; Rogers, 2004). This review examines these techniques and describes their application to soils and sediments.

METHODOLOGIES

The basic methodology for FISH-MAR in soils and sediments can be divided into five steps including incubation with radioactive substrates, fixation and extraction, FISH, MAR, and microscopy (visualization). Variations in these methodologies are discussed to facilitate effective adaptation of these techniques.

Incubation with Radioisotopic Compounds

The FISH-MAR technique can be used to identify the general metabolic activity of a microbial community (Nielsen et al., 2003a), to study the in situ physiology of a defined (probe-specific) groups of microorganisms (Gray et al., 2000), or to identify and quantify microorganisms responsible for a physiological process of interest in the environment (Nielsen and Nielsen, 2002; Gieseke et al., 2005). Incubation conditions and radioactive substrates are chosen depending on the objective of the study. For instance, substrates such as amino acids, glucose, and acetate have been used to identify overall metabolic activity, and may be more sensitive than alternative methods to detect viability such as reduction of the redox dye 5-cyano, 2,3-tolyl-tetrazolium chloride (Vollertsen et al., 2001; Nielsen et al., 2003b). Leveraging the use of these or more specific substrates with controlled incubation conditions has also proven useful for identifying the metabolic activity of specific functional groups such as autotrophic, mixotrophic, and heterotrophic bacteria under aerobic, denitrifying, Fe-reducing, and sulfate-reducing conditions in complex communities (Ito et al., 2002; Nielsen and Nielsen, 2002; Nielsen et al., 2002; Thomsen et al., 2004; Güven et al., 2005). Microbial processes have also been studied with more specific substrates such as radioisotopic orthophosphate for quantifying phosphate-accumulating organisms (Kong et al., 2002, 2004; Lee et al., 2003).

The incubation period is a critical factor in study design. Where the intrinsic microbial community structure is of interest, incubation periods must be minimized to reduce potential divergence caused by the selective pressures of incubations. Enough time must be given, however, to allow sufficient absorption or cellular incorporation of the radioisotope (but not necessarily cell doubling) to detect cells using microautoradiography. Therefore, the appropriate incubation conditions can vary depending on the metabolic activity of the community of interest and the substrate used, and will rarely be known a priori. For more active microbial communities such as those derived from wastewater treatment plants or natural surface waters, short incubation times, generally ranging from 2 to 4 h, have been reported (Andreasen and Nielsen, 1997; Lee et al., 1999; Kindaichi et al., 2004; Ginige et al., 2004). In less active microbial communities or those consuming more challenging substrates such as xenobiotic compounds, longer incubation periods of up to 2 d have been required to achieve visible autoradiograms (Yang et al., 2003; Rogers, 2004). Due to these uncertainties, each new study should investigate the effects of incubation time on both the autoradiographic response and divergence of microbial communities from their initial structure.

Cell Fixation and Extraction

Cell fixation stabilizes and permeabilizes microbial cells for later introduction of oligonucleotide probes during the FISH step. Fixation can be performed following extraction of cells from the soil or directly in the soil matrix. Solutions of paraformaldehyde or formaldehyde with dehydrating ethanol washes are common fixatives. Modifications of these procedures to optimize staining of Gram-positive microorganisms include ethanol and lysozyme pretreatments to increase cell permeability and reduce potential cross-linking of paraformaldehyde that can lower cell-wall permeability, and are described by Moter and Göbel (2000). It appears unlikely that a single fixative will achieve optimal results for all cell types. For mixed microbial communities, however, Hugenholtz et al. (2001) suggested that paraformaldehyde fixation will yield satisfactory results.

In some instances, the association of microbial cells with the soil matrix is of interest. In these cases, differential extraction techniques of increasing rigor can be used to identify less tightly bound cell types apart from those that are closely associated with soil particles (Ranjard et al., 2000). Further, using CLSM and fluorescent dyes, it is possible to visualize spatial distributions of microorganisms directly in association with soil aggregates (Li et al., 2004). Although FISH has not yet been applied in such a fashion, it has been shown to be useful for visualizing bacterial cells bound to soil particles (Christensen et al., 1999), and therefore should be amenable to similar studies. Alternatively, stabilizing agents such as agarose or epoxy resins may aid in the study of microbial structure in relation to soil aggregates, but have yet to be explored in detail with FISH methods (see discussion below). Advances in probe technologies, fluorescence microscopy, and digital imaging should focus on reducing background fluorescence of soils and related organic materials, and increasing microscopic resolution.

If the intact structure of the microbial community is of less interest than identifying and quantifying the community composition, then extraction of cells from the soil matrix can increase the sensitivity of the FISH technique considerably. Extraction of microorganisms from the soil matrix includes reduction in background fluorescence, allowing the detection of microorganisms that would otherwise be blocked from view by soil particles, and the concentration of microorganisms, increasing the probability of detecting groups of microorganisms present in smaller populations. Therefore, microbial cell extraction can be an important aspect in the application of FISH to soil and sediment samples. Ideally, methods should maximize recovery of intact cells, remove interfering mineral or organic substances that can affect background fluorescence, and result in a community structure equivalent to that of the parent soil or sediment.

Cell extraction methods were originally developed for direct microscopic cell counting. Known volumes of diluted soil or water were spread directly on slides (the soil smear) and stained (Jones and Mollison, 1948) or passed through filters (Hobbie et al., 1977). Further modifications (extractants, centrifugation steps) were tested in the course of ecological studies of *Rhizobium* and other bacteria using fluorescent antibodies (Bottomley, 1994). A variety of extraction solutions have been commonly included phosphate buffer, sodium pyrophosphate, cation exchange resins, and detergents such as Tween (polysorbate). These solutions, when combined with mechanical agitation or sonication, act to disperse soil microaggregates and release the bacteria. Lindahl and Bakken (1995) found that shaking and sonication resulted in greater cell recoveries than blenders or other harsh mechanical treatments and recommended extraction solutions with a pH of 8 to 9. More recently, a method has been proposed for marine sediments that used 10% methanol

as an extractant (Lunau et al., 2005). The methanol extractant was superior to pyrophosphate and Tween 80 based extractions in marine sediments. We are not aware of the use of this method in soils. The soil smear technique has been used in a number of recent studies (Kobabe et al., 2004; Nogales et al., 2001; Zarda et al., 1997). The advantages of the soil smear technique are simplicity and ease.

Extraction procedures followed by density gradient centrifugation have also been widely used. These methods use soil dispersion followed by low-speed centrifugation or a settling period to remove sand and silt particles. Nycodenz gradients, developed by Lindahl and Bakken (1995) appear to be effective for soils and sediments. Extracted cells can be applied directly to a Nycodenz gradient (Axis-Shield PoC AS, Oslo, Norway) and concentrated by high-speed centrifugation, effectively removing clays and aggregating bacteria near the Nycodenz–water interface. Centrifugation-based methods have the potential to concentrate cells and remove soil and organic materials that contribute to background autofluorescence, which should allow easier detection of smaller populations. Polyvinylpyrrolidione can be added to extraction solutions to remove humic acids, which contribute to background autofluorescence. Treatment of extracts with 0.01% (w/v) of toluidine blue has been reported to reduce background fluorescence of soil particles (Weber et al., 2001).

Fluorescent In Situ Hybridization

The FISH technique relies on specific binding of a constructed oligonucleotide probe complimentary to the rRNA of an organism of interest. Construction of an oligonucleotide probe, therefore, requires at least partial knowledge of the rRNA sequence of the target cell. The most common target sequences are within the 16S subunit of the bacterial ribosome, but other ribosome subunit sequences have also been used.

Oligonucleotide probes typically consist of 18 to 30 nucleotide bases conjugated to a fluorescent marker on the 5′ end that allows detection of probes bound to cellular rRNA by fluorescence microscopy. By conjugating probes with different fluorochromes to selected microbial targets, several target organisms can be visualized at the same time. Probe fluorochromes should be chosen to yield the largest degree of contrast to potential autofluorescence from soil components.

Probes suitable for use in FISH have been developed at a variety of taxonomic levels (Table 1). At the domain level, the EUB338 probes (I, II, and III) target the Bacteria, while the Archaea are stained by the ARCH915 probe and the Eukaryota by the EUK516 probe. More specific probe sets for lower taxonomic levels are described in published literature, or can be constructed using consensus rDNA

Table 1. Examples of group-specific oligonucleotide probes relevant to soil microbial communities.†

Target organisms	Probe	Coverage ‡	Mismatch ratio §	Oligonucleotide sequence (5′– 3′)	Reference
		%	%		
Universal	UNIV1390	92	— ¶	GAC GGG CGG TGT GTA CAA	Zheng et al. (1996)
Eukarya	EUK516	NR#	NR	ACC AGA CTT GCC CTC C	Amann et al. (1990)
Archaea	ARCH915	81	NR	GTG CTC CCC CGC CAA TTC CT	Stahl and Amann (1991)
Crenarchaeota	CREN512	76	NR	CGG CGG CTG ACA CCA G	Jurgens et al. (2000)
Euryarchaeota	EURY514	69	NR	GCG GCG GCT GGC ACC	Jurgens et al. (2000)
	EUB338	90	—	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
Bacteria	EUB338-II	0.7	—	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
	EUB338-III	1.0	—	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
Acidobacteria	HoAc1402	95	104	CTT TCG TGA TGT GAC GGG	Juretschko et al. (2002)
Actinobacteria	HGC69a	NR	NR	TAT AGT TAC CAC CGC CGT	Roller et al. (1994)
Proteobacteria					
Alphaproteobacteria	ALF968	79	19	GGT AAG GTT CTG CGC GTT	Neef (1997)
Betaproteobacteria	BET42a	NR	NR	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
Gammaproteobacteria	GAM42a	NR	NR	GCC TTC CCA CAT GCT TT	Manz et al. (1992)
Deltaproteobacteria	SRB385	34	203	CGG CGT CGC TGC GTC AGG	Amann et al. (1990)
Verrucomicrobia	EUB338-III	93	97	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
Bacteroidetes	CF319a	38	3.1	TGG TCC GTG TCT CAG TAC	Manz et al. (1996)
Bacteroidetes	BAC303	64	0.6	CCA ATG TGG GGG ACC TT	Manz et al. (1996)
Flavobacteria	CFB563	84	9.1	GGA CCC TTT AAA CCC AAT	Weller et al. (2000)
	LGC354a	7	0.1	TGG AAG ATT CCC TAC TGC	Meier et al. (1999)
Firmicutes	LGC354b	21	0.2	CGG AAG ATT CCC TAC TGC	Meier et al. (1999)
	LGC354c	17	0.1	CCG AAG ATT CCC TAC TGC	Meier et al. (1999)
Chloroflexi	GNSB941	94	66	AAA CCA CAC GCT CCG CT	Gich et al. (2001)
Chloroflexi	CFX109	46	3.9	CAC GTG TTC CTC AGC CGT	Björnsson et al. (2002)
Anaerolineae	CFX784	40	11	ACC GGG GTC TCT AAT CCC	Björnsson et al. (2002)
	EUB338-II	69	6.8	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
Planctomycetes	PLA46	75	52	GAC TTG CAT GCC TAA TCC	Neef et al. (1998)
	PLA886	74	5.0	GCC TTG CGA CCA TAC TCC C	Neef et al. (1998)
Nonsense control	NON338	<0.01	$1.5 \cdot 10^6$	ACT CCT ACG GGA GGC AGC	Wallner et al. (1993)

† For a more comprehensive list of group-specific probes, see probeBase (http://www.microbial-ecology.net/probebase/default.asp [verified 16 Dec. 2006]; Loy et al., 2003).

‡ The percentage of sequences within the target group that show a full match to the probe sequence. Databases used included the Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp [verified 16 Dec. 2006]; Cole et al., 2005) and the ARB Project (http://www.arb-home.de/ [verified 16 Dec. 2006]; Ludwig et al., 2004).

§ The ratio of sequences outside the target group to the sequences inside the target group that exhibit a full match to the probe sequence (expressed as a percentage).

¶ No mismatches.

Not reported due to insufficient data.

data published on large public access databases (e.g., the Ribosomal Database Project II [Cole et al., 2005]). Detailed protocols for the design and evaluation of 16S-rRNA-targeted oligonucleotide probes for use in environmental samples are available (Hugenholtz et al., 2001). Alternatively, a useful public access database for selection of FISH probes at various taxonomic levels that have been cataloged from published literature is probeBase (http://www.microbial-ecology.net/probebase/default.asp [verified 16 Dec. 2006]; Loy et al., 2003). As new 16S rDNA sequences and oligonucleotide probes are continually identified and added to public access databases, the consensus sequences need to be periodically checked.

The FISH technique is performed following cell extraction and fixation. The chosen oligonucleotide probes are applied in solution to the soil or soil extract and allowed to hybridize to complementary rRNA of the cells of interest. Hybridization and subsequent wash solutions use temperature, NaCl, and formamide to control stringency. These conditions vary from probe to probe and optimal conditions maximize probe binding while minimizing nonspecific binding (Bouvier and del Giorgio, 2003). Detailed protocols for FISH are available in published literature (Hugenholtz et al., 2001; Daims et al., 2005).

Methods used in FISH-MAR for oligonucleotide staining are generally similar to those described above. The most important consideration is performing the FISH procedures before MAR so that silver granules deposited adjacent to radioactive cells during MAR are not dislodged during the hybridization steps (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000). Of further concern is the ability to visualize cells that may be masked behind dense silver grain formations. To overcome this limitation, cells can be fixed to gelatin-, poly-L-lysine-, or 3-aminopropyltriethoxysilane-coated microscope cover slides by drop mounting or transferring from polycarbonate filters using a sandwich technique before FISH and MAR (Lee et al., 1999; Cottrell and Kirchman, 2000; Nielsen et al., 2003a). In this way, cells are located between the microscope objective lens and the underlying silver grain clusters, making them visible with fluorescence microscopy when mounted onto microscope slides following MAR. Alternatively, FISH can be performed on polycarbonate filters, which are then placed cell-side down onto autoradiographic-emulsion-coated slides. The filter is then peeled away following slide development (Malmstrom et al., 2004). Greater than 80% retention of cells to slides can be achieved using these techniques (Malmstrom et al., 2004). Cells should be extracted from soils before FISH-MAR because soil particles can interfere with resolving silver grain density images. Soils may also bind hydrophobic radioisotopic substrates, yielding false-positive results or severe autoradiographic background signals.

A common difficulty for the application of FISH to soils and sediments is a low signal-to-noise ratio. This may be caused by excessive background fluorescence from soils, sediments, or plant materials that are co-located or co-extracted with microbial cells or low activity of cells (low numbers of ribosomes per cell) that affects the total number of fluorochromes (fluorescence intensity) per cell. Aside from differential staining techniques and cell extraction methods described above to reduce background fluorescence, several strategies have been proposed that increase the probe specificity or fluorescence signals of FISH probes to cope with these issues. The simplest solutions include the use of newer marker dyes including the Cy dyes (especially Cy3), Alexa dyes, FAM, and TAM. These markers have advantages over older fluorescein (FITC, FLUOS) and rhodamine (TRITC) derivatives of being brighter and having less tendency to fade during the microscopic examination. Another simple solution is attaching a fluorochrome at both the 3′ and 5′ ends of the oligonucleotide probe to effectively double the fluorescence signal.

There are several other options for increasing the fluorescence of stained cells in soil- or sediment-derived samples. Pernthaler et al. (2002) compared polyribonucleotide probes to oligonucleotide probes in enumeration of Bacteria and Archaea. Polyribonucleotide probes are long (>100 bases) rRNA transcripts of 16S rDNA that contain numerous fluorochrome-labeled bases. The polyprobes have the advantage of greater fluorescence, but require careful fixation and hybridization to allow these larger molecules to enter the cells. Another approach, catalyzed reporter deposition (CARD-FISH), uses tyramide signal amplification (TSA) following hybridization of horseradish peroxidase-linked oligonucleotide probes to 16S rRNA in target cells. The peroxidase acts on fluorochrome tyramides to leave deposits of fluorochrome adjacent to the oligonucleotide inside the target cell (Bobrow et al., 1989; Pernthaler et al., 2002). The advantage of this technique is increased brightness, which may contribute to an increased ability to detect populations. The fixation and hybridization procedures may require modification to allow these larger molecules to enter the cells. A unique adaptation of the TSA technique is the use of quantum dots (nanocrystals with a CdSe core and ZnS shell coated with a hydrophilic polymer layer) in lieu of traditional fluorochromes, which results in a much brighter and more photostable stain (Ness et al., 2003; Alivisatos et al., 2005). Although the joint application of CARD-FISH and quantum dots has yet to be reported, there are no apparent reasons why these technologies cannot be combined to improve fluorescence signals in a FISH format. Peptide nucleic acid probes (PNA), which contain a polyamide backbone instead of the sugar-phosphate component of nucleic acids, are another alternative to the conventional oligonucleotide. These probes are reported to be more stable and bind more strongly than oligonucleotides (Lehtola et al., 2005). Finally, PNA molecular beacon probes offer an improved signal-to-noise ratio compared with DNA oligonucleotide probes (Xi et al., 2003). These probes are designed to take advantage of fluorescence resonance energy transfer by folding into a hairpin loop at low temperatures, bringing into close contact the fluorochrome and a quencher molecule, which are covalently linked to the 5′ and 3′ ends of the oligonucleotide probe. When applied at high temperature and hybridized to target RNA, the probes are unfolded, thus maximizing the distance between the fluorochrome and quencher molecule and resulting in a bright signal when excited at the appropriate wavelength. The inclusion of a hairpin loop in probe design provides an intrinsic competitor for probe binding, increasing probe specificity to complimentary rRNA.

Microautoradiography

Microautoradiography relies on the detection of decaying radioisotopic compounds via exposure of a thin layer of highly sensitive photographic emulsion. Briefly, radioactive cells release beta decay particles into the emulsion during exposure, which reduce the silver ions in silver halide crystals to silver atoms. When developed, additional reduction of silver ions occurs preferentially where atomic silver is already present, resulting in small "clumps" of silver metal (silver grains) adjacent to radioactive cells. These silver grains can be seen clearly by transmission light microscopy.

Microautoradiography should be performed in complete darkness and according to the manufacturer's instructions. To reduce film thickness and the density of silver grains (making microscopic visualization more manageable), however, autoradiography emulsion is typically diluted up to 1:3 with ultrapure water, gelatin, or agarose before use (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Teira et al., 2004; Alonso and Pernthaler, 2005). Care must be taken to not thin the film too much, which can result in uneven distribution over bioflocs or smaller cell aggregates (Nielsen et al., 2003a). Quality control procedures for microautoradiography should include: (i) replicates of each sample, (ii) positive and negative control strains for uptake of the radioisotope(s) of interest, (iii) paraformaldehyde-fixed or pasteurized samples incubated with the radioisotope(s) of interest to check for adsorption phenomena, and (iv) samples incubated without added radioactivity to check for chemography.

Exposure times for microautoradiography cannot be predicted a priori, as they can range from a few hours to weeks depending on several factors including, but not limited to: (i) the decay rate of the radioisotope used, (ii) the concentration of substrate used in the incubation, (iii) the label position and specific activity of the radioisotope, (iv) the specific cellular uptake rate, and (v) the age and dilution of the autoradiographic emulsion used. A time-course study can identify the appropriate exposure time to be used. The optimum exposure time maximizes the number of fluorescent cells that can be associated with clusters of silver grains. Clusters of silver grains are identified relative to the density of the background of silver grains (i.e., number per square micrometer), which increase with the time of exposure due to several factors including, but not limited to, the duration of exposure to decaying isotopes, incidental exposure to light, and chemography. Cells with known radioactivity can also be used as quantitative quality control standards for identifying appropriate exposure times or quantifying cell-specific substrate uptake rates (Nielsen et al., 2003a).

Microscopy

The FISH-MAR cover glasses or slides should be mounted with a low fluorescence antifade mountant before microscopic analysis. Microautoradiography-positive (MAR+) cells are counted using transmission light microscopy and probe-stained cells can be enumerated using an epifluorescence microscope or a confocal laser-scanning microscope. Switching between bright-field and fluorescence modes for each microscopic field allows MAR+ probe-defined cell types to be identified. Care must be taken to consistently identify MAR+ cells, which should be clearly distinguishable from potential background silver grain formation. No set rules for identifying silver grain clustering from background silver grain formation exist. Nielsen et al. (2003a), however, examined the density of silver grains within either 1 μm of cells consuming ^{3}H-labeled substrates or 5 μm of cells consuming ^{14}C-labeled substrates, and defined clusters of silver grains as those that were 100 times denser than the background at 10 μm from either side of MAR+ cells. Their definitions are easily adapted for use in image analysis software to automate identification of silver grain clusters by size exclusion (Rogers, 2004).

Image analysis techniques can also be used as another strategy to improve resolution and discrimination between cells and background fluorescence in FISH. These methods use charge-coupled device (CCD) cameras to capture images from the fluorescence microscope. Commercial or free software can then be used to recognize and brighten cells present on the digital images relative to the background (Schönholzer et al., 2002; Selinummi et al., 2005). The use of computer-controlled microscope stages and autofocus allows full automation of the counting process (Thiel and Blaut, 2005). Figure 1 shows an example of digital image processing for FISH-MAR. Black and white images of the same microscopic field, but from two different band pass filters (Bacteria CY3 and DAPI) are each digitally optimized for brightness and contrast, aligned by the image processing software, and assigned individual colors. These images can be composited with the complementary bright-field or phase contrast image to allow simplified detection of MAR+ cells. The green background of the autoradiogram in the composite image provides higher contrast for detecting red and blue cells.

APPLICATION TO SOILS AND SEDIMENTS

Fluorescent In Situ Hybridization

The application of FISH to soils and sediments is relatively recent compared with its use in marine and fresh water environments. Studies of community structure in soils have used oligonucleotide probes (Table 1) that target relatively large groups of bacteria of phylogenetically related genera (Kobabe et al., 2004; Nogales et al., 2001; Chatzinotas et al., 1998; Zarda et al., 1997). Zarda et al. (1997) reported that of EUB338-stained cells in soil, the Alphaproteobacteria and Planctomycetes were the most dominant groups of those investigated in their study. Chatzinotas et al. (1998) applied probes representing seven phyla and subphyla of bacteria to investigate microbial community structure of pristine forest soils and reported that Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria (SRB385), and Planctomycetes were dominant. These researchers also determined, however, that detection of actinomycetes in soils may be affected by extraction procedures that include a sonication step. Of six bacterial phyla and subphyla investigated in a subsurface artic tundra soil, Kobabe et al. (2004) reported that the most dominant groups were the Betaproteobacteria and the Cytophaga–Flavobacterium. Caracciolo et al. (2005) examined the shifts in community structure caused by the herbicide simazine or urea additions to soil. Of the groups they studied, the Alphaproteobacteria, Betaproteobacteria, and Planctomycetes were most dominant in the untreated soil. The Alphaproteobacteria and Betaproteobacteria were most affected by the treatments, but the significance of these shifts in community structure was not determined. Based on examination of 32 different 16S rRNA and rDNA clone libraries containing a total of 3398 bacterial clones obtained from various soil environments globally, Janssen (2006) showed that 92% could be classified into one of nine phyla: Proteobacteria (39.2%), Acidobacteria (19.7%), Actinobacteria (12.7%), Verrucomicrobia (7.0%), Bacteroidetes (5.0%), Chloroflexi (3.2%), Planctomycetes (2.0%), Gemmatimonadetes (2.0%), and Firmicutes (1.8%). These findings support the selection of group-specific probes for exploration of soil microbial communities in the FISH studies reported above. Aside from bacteria, Zarda et al. (1997), Chatzinotas et al. (1998), and our own work with subsurface sediments (Rogers, 2004) indicate that

Archaea in soils account for only a small fraction of the total population (<5%).

The FISH technique has also been used to investigate the structure of smaller microbial communities or visualize more specific microbial targets, some of which belong to bacterial phylotypes that have not been previously cultivated. For example, Eller et al. (2001) described the development and application of probes specific for two groups of methanotrophs. They used multiple fluorochromes and image overlapping to visualize both groups of methanotrophs simultaneously in soil. Ludwig et al. (1997) extracted and polymerase chain reaction (PCR) amplified rDNA from soil, and then cloned several of the PCR products into *Escherichia coli*. Comparison of these cloned sequences to databases of other rDNA sequences indicated that 50 were representative of a bacterial group containing *Holophaga*, *Geothrix*, and *Acidobacterium*. Specific oligonucleotide probes were constructed, and cells from soil extracts were visualized by FISH.

A unique application of the FISH technique is to examine the spatial relationships of different groups of microorganisms and their comparative abundance in relatively undisturbed samples. Both FISH and CLSM have been used to examine rhizosphere populations of various microorganisms (Assmus et al., 1995; Gilbert et al., 1998; Watt et al., 2006). Stabilization of the physical structure of the sample may be necessary. Macnaughton et al. (1996) used agarose as a stabilizing agent after fixation with paraformaldehyde for FISH analysis of rhizosphere populations, allowing more precise visualization of the distribution of bacteria on plant roots. Epoxy resins have also been used to structurally stabilize samples before examination with FISH (Moter et al.,1998). The use of FISH with resin-embedded soil samples has not yet been systematically examined, but Li et al. (2004) have reviewed many aspects of preparing soil thin sections and staining procedures. Nunan et al. (2001, 2002) prepared epoxy resin thin sections of soil prestained with calcafluor white instead of FISH to explore the spatial variation in bacterial populations in relation to soil aggregate and pore structure. They noted differences between topsoils and subsurface soils in bacterial aggregation related to pore structure. Alternatively, differential cell extraction procedures have also been shown effective to examine microbial community structure in parts of soil microaggregates (see above).

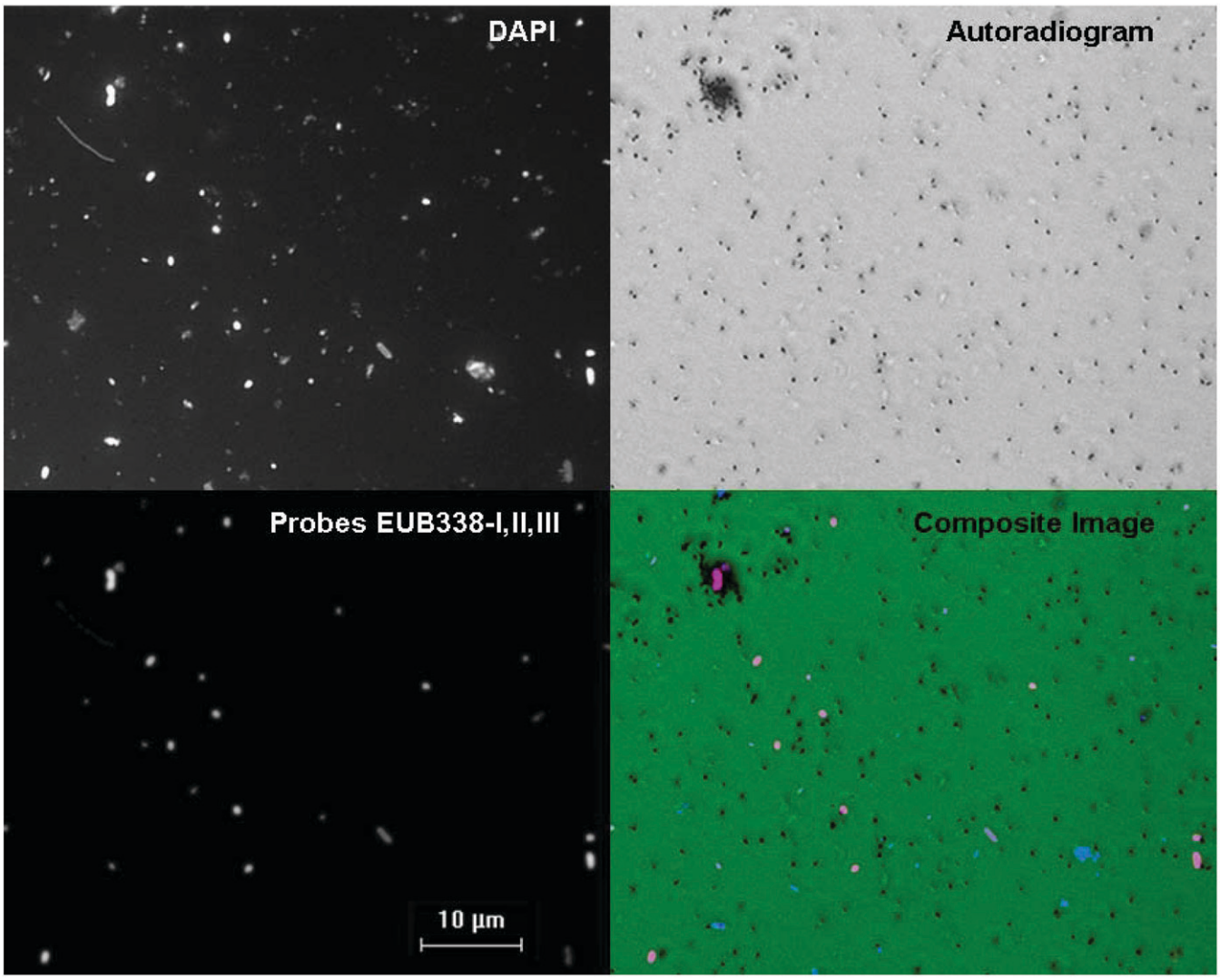

Fig. 1. Digital image processing of fluorescent in situ hybridization (FISH)–microautoradiography (MAR). Separate images for each color channel and the phase contrast counter image can be composited into a single image to simplify detection of cells that consume radioisotopic substrates. Stain DAPI (excitation = 385–400 nm, emission = 450–465 nm); probe EUB338-I, II, III (stain CY3; excitation = 530–560 nm, emission = 573–648 nm).

A potential limitation for the use of FISH in environmental matrices, including soil, is that Eubacteria and Archaea probe-detected cells are typically one- to two-thirds that of total cell counts obtained by the commonly used counterstains, acridine orange, SYBR Green, or DAPI, which bind nonspecifically to cellular DNA (Bouvier and del Giorgio, 2003; Eller and Frenzel, 2001; Kobabe et al., 2004; Nogales et al., 2001; Stein et al., 2005; Zarda et al.,1997, 1998; Chatzinotas et al., 1998). Bouvier and del Giorgio (2003) attributed the difference between EUB338- and DAPI-stained cells in aquatic systems to low-intensity fluorochromes, inefficient fixation and hybridization procedures, and low cellular ribosome counts. These factors operate independently or in combination to produce poorly stained cells and undercounted populations, which in turn contribute to the discrepancy between DAPI and EUB338 cell counts in soils. Strategies for overcoming some of these problems are discussed above. Natural environments are also likely to harbor substantial populations detectable by DAPI, but not by EUB338 probes, such as Eukaryotes and Planctomycetes (Neef et al., 1998). Of the group-specific probes presently available for FISH, the within-group coverage ranges from 34 to 95%, potentially resulting in many false negative results. Better probe designs could alleviate some of these issues, but soils may still harbor several uncultured and unsequenced bacteria that are detected by DAPI but not by the EUB338 probe set. Finally, Zweifel and Hagström (1995) and Luna et al. (2002) showed evidence that up to 70% of DAPI-stained cells could be dead or inactive, potentially as a result of DAPI binding to sites created by paraformaldehyde fixation. A more vigorous destaining step may improve discrimination of viable and nonviable cells (Zweifel and Hagström, 1995).

Fluorescent In Situ Hybridization–Microautoradiography

The FISH-MAR technique is emerging as a powerful tool for environmental microbiologists, enabling identification of microorganisms catalyzing specific processes in various environments and the study of the physiology of defined groups of microbes in situ. For instance, FISH-MAR has proven useful for improving cultivation strategies for bacterioplankton from lake waters and studying the in situ distribution and substrate uptake patterns of *Chloroflexus* spp. in hot-spring microbial mats (Vollertsen et al., 2001; Nübel et al., 2002; Bruns et al., 2003). Although broad in potential, the overwhelming majority of reported applications of FISH-MAR to date have focused on nutrient removal and process control in wastewater treatment and, to a lesser degree, the characterization of natural microbial communities in marine and estuary environments. Many of these applications have been tabulated in previous reviews (Gray and Head, 2001; Wagner and Loy, 2002; Wagner et al., 2006). The paucity of reported applications of FISH-MAR to soils and sediments is perhaps related to the difficulties associated with application of FISH to these matrices, as described above. Using appropriate adaptations to FISH protocols, however, as discussed above, similar objectives could be addressed in regard to soil microbial communities.

For instance, the biogeochemical cycling of N is an important process in soils and aquatic ecosystems affecting soil fertility, plant growth, and water quality. Nitrogen removal is also an important process in wastewater treatment for protection of water quality. Nutrient removal during wastewater treatment relies on nitrification of NH_4^+ and subsequent denitrification before discharge. Since establishment and maintenance of nitrification in wastewater treatment can be a lengthy and difficult process, there is intense interest in understanding the ecophysiology of nitrifying bacteria that may lead to more stable system performance. The FISH-MAR technique has been used extensively to study the ecophysiology of nitrifying bacteria in wastewater treatment systems, and to identify dominant groups of denitrifying bacteria in mixed microbial communities of bioreactors (Daims et al., 2001; Kindaichi et al., 2004; Gieseke et al., 2005; Ginige et al., 2004; Okabe et al., 2005). Adapting the methodologies of these researchers to study plant–microbe interactions in the root zone or other soil systems should be rapid, and has long been a desirable capability. In fact, before the discovery of FISH techniques, Fliermans and Schmidt (1975) were able to study the ecophysiology of *Nitrobacter agilis* and *N. winogradskyi* in soils from Mammoth Cave using fluorescent antibody staining combined with MAR.

Another application of FISH-MAR is the identification microorganisms catalyzing the biodegradation of xenobiotic organic contaminants in polluted soils and quantification of their intrinsic activity. Prediction of biodegradation and transformation processes in soils, subsoils, and aquifer materials is limited by our knowledge of the in situ populations that are active in the biodegradation process, and the interaction of these communities with the environmental conditions. A variety of 16S rDNA analyses indicate that microbial communities in aquifer sediments are diverse and can be dynamic in space and time. Complex food webs may result from the introduction of large, localized C sources into soils or aquifers through pollutant spills or leaks (Carman et al., 1995; Ghiorse et al., 1995; Langworthy et al., 1998).

In a recent work, we applied FISH and MAR to characterize the microbial community of a coal-tar-impacted aquifer in which polycyclic aromatic hydrocarbons (PAHs) were the primary pollutants (Rogers, 2004). In this aquifer, the microbial community was three orders of magnitude greater in population in the coal-tar source region than in nearby unaffected sediments. Spatial heterogeneity in the intrinsic microbial community structure was observed. Actinobacteria, Gammaproteobacteria, Bacteroidetes, and Betaproteobacteria dominated the aerobic (>1 mg/L dissolved O_2) and NO_3–reducing in situ microbial community. Sulfate-reducing bacteria (SRB385, Table 1) were dominant in sediments associated with sulfate reduction. Using FISH-MAR, the uptake of [UL-^{14}C]naphthalene and [9-^{14}C]phenanthrene under aerobic conditions were associated with members of the Betaproteobacteria, Gammaproteobacteria, and Actinobacteria (see Fig. 2). The number of bacteria taking in naphthalene ranged from 2.4 to 4.5% of the total microbial community (1.0×10^7 to 2.2×10^8 naphthalene-degrading bacteria/kg sediment), and included all three taxa regardless of the sediments investigated. The number of bacteria taking in phenanthrene ranged from 0.8 to 1.0% of the total microbial community (8.0×10^6 to 4.0×10^7 phenanthrene-degrading bacteria/kg sediment), but exhibited differences in the uptake patterns by the Betaproteobacteria and Gammaproteobacteria depending on the aquifer sediments investigated. The combination of FISH and MAR showed that only a small fraction of the intrinsic microbial community could be

directly related to the uptake of naphthalene and phenanthrene. This is contrary to current models of biodegradation, which assume a single physiology and constant activity for PAH-degrading bacteria. Direct evidence of intrinsic degradation of PAH pollutants such as that provided by FISH-MAR may be necessary to construct better predictive models of intrinsic biodegradation at complex PAH-contaminated sites (Smets and Pritchard, 2003).

Limitations of Fluorescent In Situ Hybridization–Microautoradiography

As shown above, FISH-MAR can be a powerful technique for linking the identity and function of complex microbial communities in situ. The FISH-MAR technique can only be used on samples in which the substrate of interest is one that will be incorporated into the biomass, thus making it capable of producing a MAR signal. Interpretation of FISH-MAR assays may also be complicated in instances where multiple degradation steps are catalyzed by different microorganisms or where alternative substrates are plentiful. The use of multiple labeled substrates representing each step in the degradation pathway may yield more useful information regarding the in situ physiology of microbial communities.

Environmental samples with low microbial activities may be problematic for FISH-MAR because they may contain cells of insufficient 16S rRNA to be detectable with FISH or they may lack sufficient metabolic activity to elicit an autoradiographic response. To reduce false negatives, extended incubation times may be required to obtain visible cells and MAR density images; however, this may lead to divergence from the intrinsic microbial community structure and activity. Furthermore, uptake of radioactive metabolites and cellular byproducts of growth on radioactive substrates by organisms that are not primary consumers of the substrate of interest (cross-feeding) will increase with time. For instance, heterotrophic bacteria may take in $^{14}CO_2$ produced during degradation of parent compounds for carboxylation reactions, eventually leading to false positives (Hesselsoe et al., 2005). Therefore, care must be taken to fully characterize the microbial communities before and following FISH-MAR incubations and to use appropriate controls, including negative controls, to discriminate between the true uptake of a parent isotope and experimental artifacts of the MAR procedure.

Aside from these limitations, technical advancements in FISH and MAR methodology are expanding the utility of this technique for both engineered and natural systems. For instance, the full-cycle 16S rRNA approach may be cumbersome and can underemphasize the importance of certain groups in the uptake of specific substrates in situ. Some researchers have been able to couple micromanipulation of intrinsic microbial communities to isolate and identify specific organisms of interest based on FISH in environmental samples (Thomsen et al., 2004), which could make the full-cycle approach less cumbersome. This technique has yet to be adapted, however, to the isolation and identification of MAR+ cells hybridized with group-specific probes. Finally, improvements in quantitative FISH-MAR methodology have been proposed that may offer the ability to identify in situ kinetic coefficients (Nielsen et al., 2003a). Uptake of labeled substrates was quantified by counting cell-associated silver grains after MAR and comparing with internal control standards. Measurements of uptake at different substrate concentrations allowed estimation of best-fit half

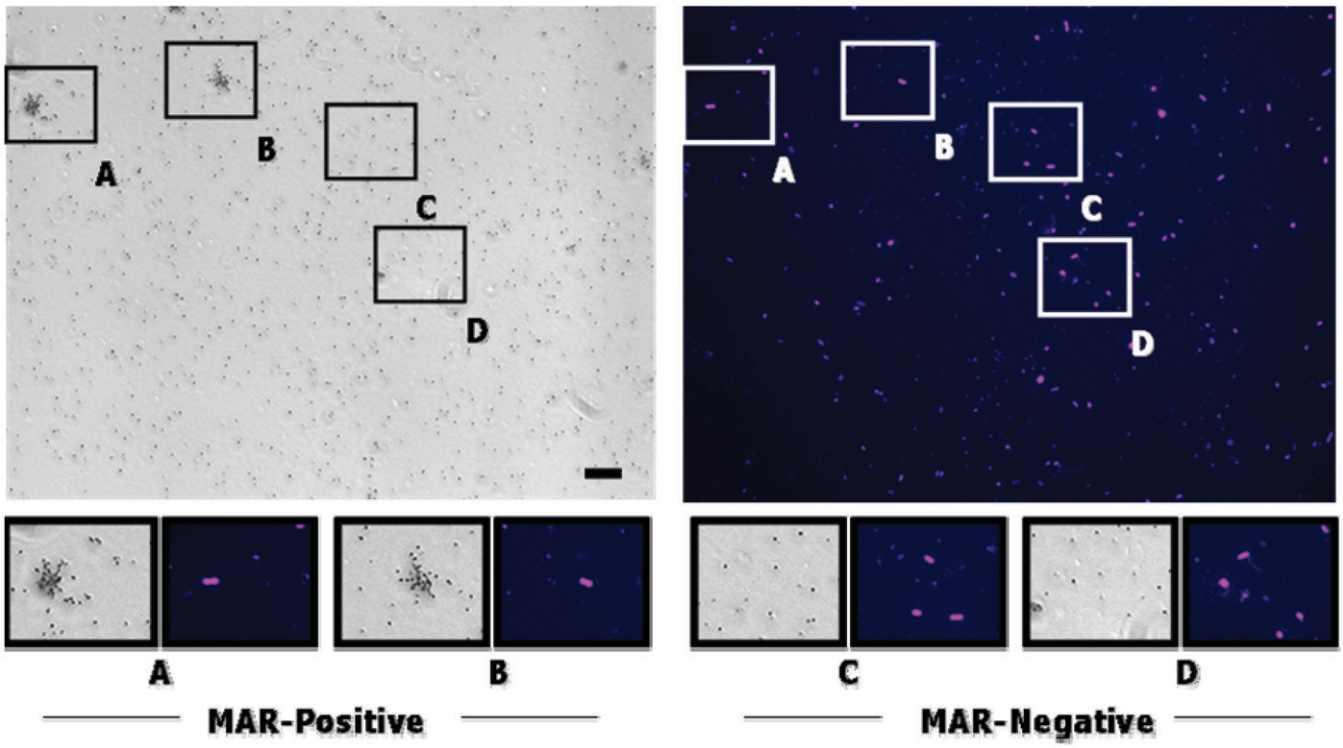

Fig. 2. Autoradiogram (left side) and corresponding fluorescence image (right side) following 10-d autoradiographic film exposure to cells from 36-h aerobic incubations of polycyclic aromatic hydrocarbon contaminated aquifer sediments with [9-^{14}C]phenanthrene. Silver grain clusters surrounding bacterial cells indicate active cellular incorporation of [9-^{14}C]phenanthrene (A and B), which can be differentiated from bacteria that do not metabolize [9-^{14}C]phenanthrene (C and D). Pink cells indicate Bacteria [EUB338(I, II, III) and blue cells are DAPI stained. Bar in autoradiogram = 10 μm. MAR = microautoradiography.

saturation constants and maximum uptake rates. Addition of a large pool of radioactive substrate at various doses to yield visible silver grain density images for microautoradiography will result, however, in a range of substrate concentrations greater than that of the original environmental sample. Use of the best-fit kinetic constants will implicitly require extrapolation of the fitted model below the range of substrate concentrations used to derive the kinetic coefficients. Interpretation of best-fit kinetic coefficients derived from quantitative FISH-MAR when applied in soils or aquifer sediments may be further complicated by processes such as diffusion or sorption that may affect uptake of radioisotopic substrates.

Complementary and Alternative Techniques

Several techniques can provide alternative or complementary information on the microbial community structure of soils. For instance, Buckley and Schmidt (2003) extracted rDNA followed by quantitative dot-blot hybridization with ^{32}P-labeled oligonucleotide probes to obtain information that was qualitatively similar to information that could have been obtained by a FISH procedure. Nogales et al. (2001) compared community structure assessed through 16S rDNA and rRNA sequence libraries and FISH and reported qualitative similarities between the techniques. Fierer et al. (2005) applied taxon-specific SYBR Green quantitative polymerase chain reaction (qPCR) assays to rapidly assess the relative abundance of bacteria and fungi in three soils; the results of which are very similar to those for which FISH was used for identifying microbial community structure reported above. Clearly, a variety of rDNA based techniques (qPCR, terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, and ribosomal intergenic spacer analysis) have been applied to soil, but recovery of DNA, differences in copy numbers of DNA between bacterial cell types, and subsequent PCR amplification steps introduce potential bias. Construction of sequence libraries can offer more detailed views of community structure and diversity (e.g., Ranjard et al., 2000; Zhou et al., 2004) than those obtained with a limited set of FISH probes, but these efforts may require a greater investment of resources.

The FISH-MAR technique provides information on both community structure and function. At present, the number of microbial cell types that can be detected at once is effectively limited by the number of fluorochromes that can be simultaneously resolved using CLSM. This may result in tedious repetition, especially when attempting to identify microorganisms catalyzing specific processes in natural environments. Advanced beta imaging technologies allow the visualization of radioactivity with a spatial resolution as low as 20 µm. Although this resolution is not fine enough to replace MAR for co-locating FISH-detected cells in a whole-cell format, it does allow for construction of isotope arrays that can be used in a PCR-independent manner to rapidly screen microbial communities of various habitats to identify microorganisms consuming a specific substrate of interest (Adamczyk et al., 2003). Briefly, following incubation with radioisotopic substrates similar to FISH-MAR, whole-community rRNA is extracted, fragmented, conjugated to a fluorescent dye, and hybridized to an rRNA-targeted microarray. A fluorescence scanner and beta imager are used to quantify probe signal and radioactivity (Wagner et al., 2006). The effectiveness of the isotope array approach will depend on the availability of suitable rRNA-targeted oligonucleotide microarrays.

Stable isotope techniques comparable to FISH-MAR include stable isotope probing (SIP) and ^{13}C-phospholipid fatty acids analysis (^{13}C-PLFA). These techniques rely on incorporation of ^{13}C-isotopic substrates into nucleic acids or fatty acids, respectively, followed by high-speed density-gradient fractionation of heavy DNA for cloning and sequencing (SIP), or extraction and mass spectrophotometric analysis of isotopic PLFAs (^{13}C-PLFA) to identify the active microbial cell types in a variety of environments, including soil (Madsen, 2006; Griffiths et al., 2004; Butler et al., 2003). The considerations pertaining to substrate incorporation into the active microbial biomass described above are similar for ^{13}C-PLFA, SIP, and FISH-MAR. Because the extended incubation times required to achieve cell turnover critical to the SIP technique may alter the microbial community profile, FISH-MAR may be useful to validate results obtained with SIP techniques. For example, Ginige et al. (2004) used both FISH-MAR and stable isotope probing to investigate a denitrifying community in a methanol-fed bioreactor. The FISH-MAR results confirmed the identity of the dominant denitrifying microorganisms originally identified with SIP.

Time-of-flight secondary ion mass spectrometry (Cliff et al., 2002) or laser ablation mass spectrometry (Bruneau et al., 2002) in combination with microscopy to visualize cellular incorporation of the stable isotopes from substrates also yielded information on in situ microbial processes. These mass spectrophotometric techniques have been used to study soil microbial communities following dosing with ^{13}C (DeRito et al., 2005), and have been combined with FISH to identify methane-consuming Archaea in anoxic marine sediments (Orphan et al., 2001). When coupled to FISH, these techniques are subject to the same limitations in soils discussed above; however, the added capability of visualizing the ecophysiological interactions of intact soil microbial communities with their environment may yield valuable insights not obtainable with MAR or other similar isotope technologies. Aside from the practical advantages of not using radioactive substrates, stable isotopes may also offer several advantages to radioactive compounds such as their use in the field and application to N transformations. The instrumentation necessary for these techniques, however, may at present be cost prohibitive for some users.

SUMMARY

Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes is a well-established technique capable of providing information regarding the in situ phylogeny of a diverse range of microbial communities without cultivation. The strength of this technique over alternative nucleic acids technologies is that it can rapidly and specifically yield the number and spatial distribution of microorganisms in complex matrices. When combined with MAR, FISH can also yield insight into the functional activity of different microbial cell types in situ. Thus, FISH-MAR can be used to simultaneously study the microbial community structure and ecophysiology in a diverse range of habitats, and may also prove useful for improving cultivation strategies for microorganisms in complex matrices that have resisted cultivation to date (Bruns et al., 2003). Most importantly, structure–functions techniques such as

FISH-MAR may allow construction of better models of microbial processes in both natural and agricultural systems. These improved models may lead to a better understanding of soil systems.

REFERENCES

Adamczyk, J., M. Hesselhoe, N. Iversen, M. Horn, A. Lehner, P.H. Nielsen, M. Scholter, P. Roslev, and M. Wagner. 2003. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. 69:6875–6887.

Alivisatos, A.P., W. Gu, and C. Larabell. 2005. Quantum dots as cellular probes. Annu. Rev. Biomed. Eng. 7:55–76.

Alonso, C., and J. Pernthaler. 2005. Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. Appl. Environ. Microbiol. 71:1709–1716.

Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919–1925.

Amann, R.I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Andreasen, K., and P.H. Nielsen. 1997. Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. Appl. Environ. Microbiol. 63:3662–3668.

Assmus, B., P. Hutzler, G. Kirchhof, R. Amann, J.R. Lawrence, and A. Hartmann. 1995. In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl. Environ. Microbiol. 61:1013–1019.

Björnsson, L., P. Hugenholtz, G.W. Tyson, and L.L. Blackall. 2002. Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiol. 148:2309–2318.

Bobrow, M.N., T.D. Harris, K.J. Shaughnessy, and G.J. Litt. 1989. Catalyzed reporter deposition, a novel method of signal amplification: Application to membrane immunoassays. J. Immunol. 125:279–285.

Bottomley, P.J. 1994. Light microscopic methods for studying soil microorganisms. p. 81–105. *In* R.W. Weaver et al. (ed.) Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison WI.

Bouvier, T., and P.A. del Giorgio. 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): A quantitative review of published reports. FEMS Microbiol. Ecol. 44:3–15.

Bruneau, P.M.C., N. Ostle, D.A. Davidson, I.C. Grieve, and A.E. Fallick. 2002. Determination of rhizosphere ^{13}C pulse signals in soil thin sections by laser ablation isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 16:2190–2194.

Bruns, A., U. Nübel, H. Cypionka, and J. Overmann. 2003. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. 69:1980–1989.

Buckley, D.H., and T.M. Schmidt. 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ. Microbiol. 5:441–452.

Butler, J.L., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2003. Microbial community dynamics associated with rhizosphere carbon flow. Appl. Environ. Microbiol. 69:6793–6800.

Caracciolo, A.B., P. Grenni, C., R. Ciccoli, G. Di Landa, and C Cremisini. 2005. Simazine biodegradation in soil: Analysis of bacterial community structure by in situ hybridization. Pest Manage. Sci. 61:863–869.

Carman, K.R., J.W. Fleeger, J.C. Means, S.M. Pomarico, and D.J. McMillin. 1995. Experimental investigation of the effects of polynuclear aromatic hydrocarbons on an estuarine sediment food web. Mar. Environ. Res. 40:289–318.

Chatzinotas, A., R.A. Sandaa, W. Schönhuber, R. Amann, F.L. Daae, V. Torsvik, J. Zeyer, and D. Hahn. 1998. Analysis of broad-scale differences in microbial community composition of two pristine forest soils. Syst. Appl. Microbiol. 21:579–587.

Christensen, H., M. Hansen, and J. Sørensen. 1999. Counting and size classification of active soil bacteria by fluorescence in situ hybridization with an rRNA oligonucleotide probe. Appl. Environ. Microbiol. 65:1753–1761.

Cliff, J.B., D.J. Gaspar, P.J. Bottomley, and D.D. Myrold. 2002. Exploration of inorganic C and N assimilation by soil microbes with time-of-flight secondary ion mass spectrometry. Appl. Environ. Microbiol. 68:4067–4073.

Cole, J.R., B. Chai, R.J. Farris, Q. Wang, S.A. Kulam, D.M. McGarrell, G.M. Garrity, and J.M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): Sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33:D294–D296.

Cottrell, M.T., and D.L. Kirchman. 2000. Natural assemblages of marine *Proteobacteria* and members of the *Cytophaga–Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. 66:1692–1697.

Daims, H., A. Brühl, R. Amann, K.H. Schleifer, and M. Wagner. 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22:434–444.

Daims, H., J.L. Nielsen, P.H. Nielsen, K.H. Schleifer, and M. Wagner. 2001. In situ characterization of *Nitrospira*-like nitrate-oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. 67:5273–5284.

Daims, H., K. Stoecker, and M. Wagner. 2005. Fluorescence in situ hybridization for the detection of prokaryotes. p. 213–239. *In* A.M. Osborn and C.J. Smith (ed.) Advanced methods in molecular microbial ecology. Bios-Garland, Abingdon, UK.

DeLong, E.F., G.S. Wickham, and N.R. Pace. 1989. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single microbial cells. Science 243:1360–1363.

DeRito, C.M., G.M. Pumphrey, and E.L. Madsen. 2005. Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. Appl. Environ. Microbiol. 71:7858–7865.

Eller, G., and P. Frenzel. 2001. Changes in activity and community structure of methane oxidizing bacteria over the growth period of rice. Appl. Environ. Microbiol. 67:2395–2403.

Eller, G., S. Stubner, and P. Frenzel. 2001. Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence in situ hybridisation. FEMS Microbiol. Lett. 198:91–97.

Fierer, N., J.A. Jackson, R. Vilgalys, and R.B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. 71:4117–4120.

Fliermans, C.B., and E.L. Schmidt. 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with nitrobacter as a model system. Appl. Microbiol. 30:676–684.

Fuhrman, J.A., and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: Evaluation and field results. Mar. Biol. 66:109–120.

Ghiorse, W.C., J.B. Herrick, R.L. Sandoli, and E.L. Madsen. 1995. Natural selection of PAH-degrading bacterial guilds at coal-tar disposal sites. Environ. Health Perspect. 103:107–111.

Gich, F., J. Garcia-Gil, and J. Overmann. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. 177:1–10.

Gieseke, A., J.L. Nielsen, R. Amann, P.H. Nielsen, and D. de Beer. 2005. In situ substrate conversion and assimilation by nitrifying bacteria in a model biofilm. Environ. Microbiol. 7:1392–1404.

Gilbert, B., B. Assmus, A. Hartmann, and P. Frenzel. 1998. In situ localization of two methanotrophic strains in the rhizosphere of rice plants. FEMS Microbiol. Ecol. 25:117–128.

Ginige, M.P., P. Hugenholtz, H. Daims, M. Wagner, J. Keller, and L.L. Blackall. 2004. Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization–microautoradiography to study a methanol-fed denitrifying microbial community. Appl. Environ. Microbiol. 70:588–596.

Glaeser, J., and J. Overmann. 2003. The significance of organic carbon compounds for in situ metabolism and chemotaxis of phototrophic consortia. Environ. Microbiol. 5:1053–1063.

Gray, N.D., and I.M. Head. 2001. Linking genetic identity and function in communities of uncultured bacteria. Environ. Microbiol. 3:481–492.

Gray, N.D., R. Howarth, R.W. Pickup, J.G. Jones, and I.M. Head. 2000. Use of combined microautoradiography and fluorescence in situ hybridization to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus *Achromatium*. Appl. Environ.

Microbiol. 66:4518–4522.
Griffiths, R.I., M. Manefield, N. Ostle, N. McNamara, A.G. O'Donnell, M.J. Bailey, and A.S. Whiteley. 2004. $^{13}CO_2$ pulse labeling of plants in tandem with stable isotope probing: Methodological considerations for examining microbial function in the rhizosphere. J. Microbiol. Methods 58:119–129.
Güven, D., A. Dapena, B. Kartal, M.C. Schmid, B. Maas, K. van de Pas-Schoonen, S. Sozen, R. Mendez, H.J.M. Op den Camp, M.S.M. Jetten, M. Strous, and I. Schmidt. 2005. Propionate oxidation by methanol inhibition of anaerobic ammonium-oxidizing bacteria. Appl. Environ. Microbiol. 71:1066–1071.
Head, I.M., J.R. Saunders, and R.W. Pickup. 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. Microb. Ecol. 35:1–21.
Hesselsoe, M., J.L. Nielsen, P. Roslev, and P.H. Nielsen. 2005. Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$. Appl. Environ. Microbiol. 71:646–655.
Hobbie, J.E., R.J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225–1228.
Hugenholtz, P., G.W. Tyson, and L.L. Blackall. 2001. Design and evaluation of 16S rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization. Methods Mol. Biol. 176:29–42.
Ito, T., J.L. Nielsen, S. Okabe, Y. Watanabe, and P.H. Nielsen. 2002. Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic–anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Appl. Environ. Microbiol. 68:356–364.
Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728.
Jones, P.C.T., and J.E. Mollison. 1948. A technique for the quantitative estimation of soil micro-organisms. J. Gen. Microbiol. 2:54–59.
Juretschko S., A. Loy, A. Lehner, and M. Wagner M. 2002. The microbial community composition of a nitrifying–denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. 25: 84–99.
Jurgens, G., F. Glöckner, R. Amann, A. Saano, L. Montonen, M. Likolammi, and U. Munster. 2000. Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization. FEMS Microbiol. Ecol. 34:45–56.
Kindaichi, T., T. Ito, and S. Okabe. 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography–fluorescence in situ hybridization. Appl. Environ. Microbiol. 70:1641–1650.
Kobabe, S., D. Wagner, and E.-M. Pfeiffer. 2004. Characterization of microbial community composition of a Siberian tundra soil by fluorescence in situ hybridisation. FEMS Microbiol. Ecol. 50:13–23.
Kong, Y., J.L. Nielsen, and P.H. Nielsen. 2004. Microautoradiographic study of *Rhodocyclus*-related polyphosphate-accumulating bacteria in full-scale enhanced biological phosphorus removal plants. Appl. Environ. Microbiol. 70:5383–5390.
Kong, Y.H., M. Beer, G.N. Rees, and R.J. Seviour. 2002. Functional analysis of microbial communities in aerobic–anaerobic sequencing batch reactors fed with different phosphorus/carbon (P/C) ratios. Microbiology 148:2299–2307.
Langworthy, D.E., R.D. Stapleton, G.S. Sayler, and R.H. Findlay. 1998. Genotypic and phenotypic responses of a riverine microbial community to polycyclic aromatic hydrocarbon contamination. Appl. Environ. Microbiol. 64:3422–3428.
Lee, N., P.H. Nielsen, K.H. Andreasen, S. Juretschko, J.L. Nielsen, K.H. Schleifer, and M. Wagner. 1999. Combination of fluorescent in situ hybridization and microautoradiography: A new tool for structure–function analyses in microbial ecology. Appl. Environ. Microbiol. 65:1289–1297.
Lee, N., P.H. Nielsen, H. Aspegren, M. Henze, K.H. Schleifer, and J. la Cour Jansen. 2003. Long-term population dynamics and in situ physiology in activated sludge systems with enhanced biological phosphorus removal operated with and without nitrogen removal. Syst. Appl. Microbiol. 26:211–227.
Lehtola, M.J., C.J. Loades, and C.W. Keevil. 2005. Advantages of peptide nucleic acid oligonucleotides for sensitive site directed 16S rRNA fluorescence in situ hybridization (FISH) detection of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari*. J. Microbiol. Methods 62:211–219.
Li, Y., W.A. Dick, and O.H. Tuovinen. 2004. Fluorescence microscopy for visualization of soil microorganisms—a review. Biol. Fertil. Soils 39:301–311.
Lindahl, V., and L.R. Bakken. 1995. Evaluation of methods for extraction of bacteria from soil. FEMS Microbiol. Ecol. 16:135–142.
Loy, A., M. Horn, and M. Wagner. 2003. probeBase: An online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res. 31:514–516.
Ludwig, W., S.H. Bauer, M. Bauer, I. Held, G. Kirchhof, R. Schulze, I. Huber, S. Spring, A. Hartmann, and K.H. Schleifer. 1997. Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol. Lett. 153:181–190.
Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar et al. 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32:1363–1371.
Luna, G.M., E. Manini, and R. Danovaro. 2002. Large fraction of dead and inactive bacteria in coastal marine sediments: Comparison of protocols for determination and ecological significance. Appl. Environ. Microbiol. 68:3509–3515.
Lunau, M., A. Lemke, K. Walther, W. Martens-Habbena, and M. Simon. 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. Environ. Microbiol. 7:961–968.
Macnaughton, S.J., T. Booth, T.M. Embley, and A.G. O'Donnell. 1996. Physical stabilization and confocal microscopy of bacteria on roots using 16S rRNA targeted, fluorescent-labeled oligonucleotide probes. J. Microbiol. Methods 26:279–285.
Madsen, E.L. 2006. The use of stable isotope probing techniques in bioreactor and field studies on bioremediation. Curr. Opin. Biotechnol. 17:92–97.
Malmstrom, R.R., R.P. Kiene, M.T. Cottrell, and D.L. Kirchman. 2004. Contribution of SAR11 bacteria to dissolved dimethylsulfoniopropionate and amino acid uptake in the North Atlantic Ocean. Appl. Environ. Microbiol. 70:4129–4135.
Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga–Flavobacter–Bacteroides in the natural environment. Microbiol. 142:1097–1106.
Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. Syst. Appl. Microbiol. 15:593–600.
Meier, H., R. Amann, W. Ludwig, and K.H. Schleifer. 1999. Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22:186–196.
Meyer-Reil, L.A. 1978. Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. 36:506–512.
Moter, A., and U.B. Göbel. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods 41:85–112.
Moter, A., G. Leist, R. Rudolph, K. Schrank, B.-K. Choi, M. Wagner, and U.B. Gobel. 1998. Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiol. 144:2459–2467.
Neef, A. 1997. Anwendung der in situ einzelzell-identifizierung von bakterien zur populations analyse in komplexen mikrobiellen biozönosen. Ph.D. diss. Technische Universität München, Munich, Germany.
Neef, A., R. Amann, H. Schlesner, and K.-H. Scheifer. 1998. Monitoring a widespread bacterial group: In situ detection of Planctomycetes with 16S rRNA-targeted probes. Microbiol. 144:3257–3266.
Ness, J.M., R.S. Akhtar, C.B. Latham, and K.A. Roth. 2003. Combined tyramide signal amplification and quantum dots for sensitive and photostable immunofluorescence detection. J. Histochem. Cytochem. 51:981–987.
Nielsen, J.L., D. Christensen, M. Kloppenborg, and P.H. Nielsen. 2003a. Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. Environ. Microbiol. 5:202–211.
Nielsen, J.L., M.A. de Muro, and P.H. Nielsen. 2003b. Evaluation of the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence in situ hybridization. Appl. Environ. Microbiol. 69:641–643.
Nielsen, J.L., S. Juretschko, M. Wagner, and P.H. Nielsen. 2002. Abundance and phylogenetic affiliation of iron reducers in activated sludge as assessed by fluorescence in situ hybridization and microautoradiography.

Appl. Environ. Microbiol. 68:4629–4636.
Nielsen, J.L., and P.H. Nielsen. 2002. Enumeration of acetate-consuming bacteria by microautoradiography under oxygen and nitrate respiring conditions in activated sludge. Water Res. 36:421–428.
Nogales, B., E.R. Moore, E. Llobet-Brossa, R. Rossello-Mora, R. Amann, and K.N. Timmis. 2001. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. 67:1874–1884.
Nübel, U., M.M. Bateson, V. Vandieken, A. Wieland, M. Kühl, and D.M. Ward. 2002. Microscopic examination of distribution and phenotypic properties of phylogenetically diverse Chloroflexaceae-related bacteria in hot spring microbial mats. Appl. Environ. Microbiol. 68:4593–4603.
Nunan, N., K. Ritz, D. Crabb, K. Harris, K. Wu, J.W. Crawford, and I.M. Young. 2001. Quantification of the in situ distribution of soil bacteria by large-scale imaging of thin sections of undisturbed soil. FEMS Microbiol. Ecol. 36:67–77.
Nunan, N., K. Wu, J.W. Crawford, and K. Ritz. 2002. In situ spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil. Microb. Ecol. 44:296–305.
Okabe, S., T. Kindaichi, and T. Ito. 2005. Fate of ^{14}C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl. Environ. Microbiol. 71:3987–3994.
Orphan, V.J., C.H. House, K.U. Hinrichs, K.D. McKeegan, and E.F. DeLong. 2001. Methane-consuming Archaea revealed by directly coupled isotopic and phylogenetic analysis. Science 293:484–487.
Otte, S., J.G. Kuenen, L.P. Nielsen, H.W. Paerl, J. Zopfi, H.N. Schulz, A. Teske, B. Strotmann, V.A. Gallardo, and B.B. Jørgensen. 1999. Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. Appl. Environ. Microbiol. 65:3148–3157.
Ouverney, C.C., and J.A. Fuhrman. 1999. Combined microautoradiography–16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. 65:1746–1752.
Pernthaler, A., J. Pernthaler, and R. Amann. 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68:3094–3101.
Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbière, J. Thioulouse, and S. Nazaret. 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA). Microb. Ecol. 39:263–272.
Rogers, S.W. 2004. Monitored natural attenuation at a coal-tar impacted aquifer in northwestern Iowa: Coupled biogeochemical and molecular microbiological approaches for establishing secondary and tertiary lines of evidence. Ph.D. diss. Iowa State Univ., Ames.
Roller, C., M. Wagner, R. Amann, W. Ludwig, and K.H. Schleifer. 1994. In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA- targeted oligonucleotides. Microbiol. 140:2849–2858. (Erratum in Microbiol. 141:1267.)
Schönholzer, F., D. Hahn, B. Zarda, and J. Zeyer. 2002. Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L. J. Microbiol. Methods 48:53–68.
Selinummi, J., J. Seppälä, O. Yli-Harja, and J.A. Puhakka. 2005. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39:859–863.
Smets, B.F., and P.H. Pritchard. 2003. Elucidating the microbial component of natural attenuation. Curr. Opin. Biotechnol. 14:283–288.
Stahl, D.A., and R. Amann. 1991. Development and application of nucleic acid probes. p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, UK.
Stein, S., D. Selesi, R. Schilling, I. Pattis, M. Schmid, and A. Hartmann. 2005. Microbial activity and bacterial composition of H_2–treated soils with net CO_2 fixation. Soil Biol. Biochem. 37:1938–1945.
Tabor, P.S., and R.A. Neihof. 1982. Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol. 44:945–953.
Tabor, P.S., and R.A. Neihof. 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl. Environ. Microbiol. 48:1012–1019.
Teira, E., T. Reinthaler, A. Pernthaler, J. Pernthaler, and G.J. Herndl. 2004. Combining catalyzed reporter deposition–fluorescence in situ hybridization and microautoradiography to detect substrate utilization by Bacteria and Archaea in the deep ocean. Appl. Environ. Microbiol. 70:4411–4414.
Thiel, R., and M. Blaut. 2005. An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. J. Microbiol. Methods 61:369–379.
Thomsen, T.R., J.L. Nielsen, N.B. Ramsing, and P.H. Nielsen. 2004. Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. Environ. Microbiol. 6:470–479.
Villa, M., R. Simó, R.P. Kiene, J. Pinhassi, J.M. González, M.M. Moran, and C. Pedrós-Alió. 2004. Use of microautoradiography combined with fluorescence in situ hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. Appl. Environ. Microbiol. 70:4648–4657.
Vollertsen, J., A. Jahn, J.L. Nielsen, T. Hvitved-Jacobsen, and P.H. Nielsen. 2001. Comparison of methods for determination of microbial biomass in wastewater. Water Res. 35:1649–1658.
Wagner, M., and A. Loy. 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. 13:218–227.
Wagner, M., P.H. Nielsen, A. Loy, J.L. Nielsen, and H. Daims. 2006. Linking microbial community structure and function: Fluorescence in situ hybridization–microautoradiography and isotope arrays. Curr. Opin. Biotechnol. 17:83–91.
Wallner, G., R. Amann, and W. Beisker. 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14:136–143.
Ward, D.M., M.M. Bateson, R. Weller, and A.L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. 12:219–286.
Watt, M., P. Hugenholtz, R. White, and K. Vinal. 2006. Numbers and locations of native bacteria on field grown wheat roots quantified by fluorescence in situ hybridization. Environ. Microbiol. 8:871–884.
Weber, S., S. Stubner, and R. Conrad. 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. 67:1318–1327.
Weller, R., F.O. Glöckner, and R. Amann. 2000. 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum Cytophaga–Flavobacterium–Bacteroides. Syst. Appl. Microbiol. 23:107–114.
Winogradsky, S. 1949. Microbiologie du sol, problemes et methods. Barneoud, Freres, France.
Xi, C.C., M. Balberg, S.A. Boppart, and L. Raskin. 2003. Use of DNA and peptide nucleic acid molecular beacons for detection and quantification of rRNA in solution and in whole cells. Appl. Environ. Microbiol. 69:5673–5678.
Yang, Y., A. Zarda, and J. Zeyer. 2003. Combination of microautoradiography and fluorescence in situ hybridization for identification of microorganisms degrading xenobiotic contaminants. Environ. Toxicol. Chem. 22:2840–2844.
Zarda, B., D. Hahn, A. Chatzinotas, W. Schöhuber, A. Neef, R.I. Amann, and J. Zeyer. 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch. Microbiol. 168:185–192.
Zarda, B., G. Mattison, A. Hess, D. Hahn, P. Hohener, and J. Zeyer. 1998. Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. FEMS Microbiol. Ecol. 27:141–152.
Zheng, D., E.W. Alm, D.A. Stahl, and L. Raskin. 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62:4504–4513.
Zhou, J., B. Xia, H. Huang, A.V. Paulumbo, and J.M. Tiedje. 2004. Microbial diversity and heterogeneity in sandy subsurface soils. Appl. Environ. Microbiol. 70:1723–1734.
Zweifel, U., and A. Hagström. 1995. Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). Appl. Environ. Microbiol. 61:2180–2185.